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THE SCREENING OF FOLATE PRODUCING LACTIC ACID BACTERIA FROM SELECTED FERMENTED TRADITIONAL FOODS AND THEIR BIOCHEMICAL AND MOLECULAR CHARACTERIZATION

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ABSTRACT

Folate, an essential B-group vitamin, plays a key role in many metabolic pathways such as energy usage, DNA and RNA biosynthesis. Humans cannot synthesize folate, so its exogenous supply either in the form of food or medicines is necessary to prevent nutritional deficiency in case of emergencies and therapy. However, a few of the recent research studies have proved that a high intake of folic acid, which is the synthetic commercially available form of folate, may cause adverse effects on the health like masking of the hematological manifestations of vitamin B12 deficiency. Thus, the only sustainable alternative is to enhance the concentration of naturally available folate in food products. The exploitation of folate producing microbes as bioreactors to enhance the natural folate levels in the foods is the call of the time.

In view of the above concept the current study was undertaken to identify and isolate those lactic acid bacteria from fermented food samples which can produce folate and increase its concentration naturally in the foods. These bacteria can be used in the bio fortification of fermented foods. Among the selected fifty isolates, forty five were considered as presumptive LAB, and were tested for their folate production. The twenty isolates which were positively screened as folate producers were biochemically characterized to evaluate their sugar utilization capacity. The top five folate producers were further identified by their 16S rRNA sequencing as species of Lactococcus and Bifidobacterium.

KEYWORDS: Microbial Folate, Lactic Acid Bacteria, Fermented Foods, Bio-Fortification & 16S rRNA Sequencing

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Original Article

INTRODUCTION

The term “Folate” refers to those forms of the water-soluble B vitamins that have the biological activity similar to folic acid. In view of its importance in metabolic processes and nutritional value it is one of the most studied enzyme. It functions in various metabolic activities like DNA synthesis, its repair, replication and methylation processes (Crittenden et al2003, Moreno et. al 2006, LeBlanc et al. 2007, Iyer 2009, Hanson and Gregory 2011, Rossi et al., 2011).

Humans have an auxotrophic requirement for folate. In spite of its availability in various foods its deficiency still exists among various population groups (Konings et al., 2001). In India also folate, vitamin B12 and iron deficiencies are predominant public health hazards (Singh et. al 2017). The Recommended Daily Allowance (RDA) of folate for adults is 0.4mg and for pregnant women it is 0.6mg (Dudeja 1997, FAO2002, Becker et. al

2004).

Diseases and Disorders Linked to Folate Deficiency

Several studies suggest that an insufficient intake of folate may lead to several disorders like Alzheimer's, osteoporosis, poor cognitive performance, increased risk of colorectal cancer, megaloblastic anemia and neural tube defects (NTD) in newborns (Melnyk et. al 1999; LeBlanc et al.2007; Luchsinger J. A 2007; Durga J. et. al 2007;Smith 2008, Katan et al. 2009; Baggott et. al 2012;Singh et. al 2017.).

To cope up with the folate deficiency among various population groups, Folic acid supplementation of certain staple foods has become mandatory in many developed as well as developing countries (Arth et al. 2016).

Adverse Effects of Synthetic Folic Acid

Folic acid has been readily used as supplemental requisite in several medicines. (Coppen, A. 2000) However, several research studies have reported adverse health effects due to high intake of synthetic folic acid that include cytotoxicity, growth promotion of already existing tumors, leukemia, arthritis, bowel cancer, ectopic pregnancies, Masking of vitamin B12 deficiency etc. (Iyer R. 2009, Saini et al. 2016). One of the health hazards that has initiated a global discussion on whether to use synthetic folate is the masking of vitamin B12 deficiency in persons who take high doses of it. (Refsum H, 2008, Shane 2003, Ulrich 2006).

Lactic Acid Bacteria as Potent Folate Producers

Natural folate produced by microorganisms is potential alternative to synthetic folic acid which has no adverse effects (Laino 2013, Iyer et al. 2009). Moreover it has been demonstrated that bacterial folate synthesized in human intestine is absorbed & used by the host (Iyer et al. 2009 Camilo 1996, Dudeja 1997, Krause 1996, Kumar 1997, Said H. M. 1997).

Lactic acid bacteria (LAB)which are diverse group of Gram positive, saccharolytic, microbes having useful properties are present in several food sources. They are known and well-studied for their array of beneficial metabolites such as bacteriocins, vitamins as well as for their probiotic characteristics (Lin and Young 2000, Rossi et al. 2011, Guarner et al 1998 & Hugenholtz et. al 2002). They can be isolated from several fermented food products like dhokla batter, idli batter, jalebi batter, lassi, yogurt, dosa batter, vegetable pickle etc (Alm 1980, Lin & Young 2000, Vijai Pal et al. 2005, Bernardeau et al. 2006, Patel et al.2012)

An important objective of the study is to screen folate producing lactic acid bacteria &identifying high folate producers from selected traditional fermented foods which are scarcely explored.

MATERIALS AND METHOD

Sample Collection

Six traditional fermented food samples consumed by the rural as well as urban population were collected from the household as well as local market. These were subjected for further analysis for screening and identification of folate producing lactic acid bacteria. All the collected samples were stored at 4^{o}C until further processing.

Isolation and Identification of Lactic Acid Bacteria (LAB)

The above collected samples were homogenized 10% (w/v) and subjected for serial dilution in sterile distilled water. 100µL of each of the diluted bacterial sample was inoculated on the Man-Rogosa-Sharpe agar (MRS agar Hi media, Mumbai) plates by Spread plate method under strict and sterile environment. The inoculated plates were further incubated at 37ºC for 48 hrs.

Pure Culture Preparation

From the master plates individual colonies with different morphological characteristics were selected and sub-cultured in MRS broth and also streaked onto MRS agar plates for obtaining pure cultures.(Stamer, 1979). All the pure culture isolates were labeled & preserved at -20ºC in MRS broth containing 15% glycerol (v/v). Working cultures were prepared by propagating them in MRS broth.

Identification of Lactic Acid Bacteria

To screen Lactic acid bacteria, the isolated pure cultures were subjected for Gram's staining, cell morphology identification and Catalase test. All Gram positive & catalase negative isolates were presumptively considered as Lactic acid bacteria.

Screening and Biochemical Characterization of Folate Producers

42Isolates selected as presumptive LAB were screened for folate production using folic acid casei medium (FAC medium, Himedia). The cultures were inoculated in 2mL of FAC medium and incubated for 18hr at 37°C. The turbidity formed because of the growth of the culture after incubation is itself an indicator of folate producers. Twenty random folate producing LAB were biochemically characterized to evaluate their sugar utilization capacity using Phenol Red Carbohydrate Broth [Trypticase: 10 g, Sodium Chloride (NaCl): 5 g, Beef extract: 1 g, Phenol red (7.2 ml of 0.25% phenol red solution): 0.018 g, Carbohydrate source: 10 g]and the folate produced by them was subjected for quantitative estimation.

Microbiological Assay for Quantification of Folate

L. casei ATCC 7469 was used as an indicator organism to perform the microbiological assay of folate produced (Horne & Patterson, 1988). The growth of L. casei is completely dependent upon the presence of folate in the medium, which is actually supplied by the folate produced by the Lactic acid test bacteria. The culture was incubated at 37°C for 48 hr. in the dark. The rate of growth of bacteria is directly proportional to the presence of folate in the medium. The bacterial growth is calculated based on turbidity measured using UV Spectrophotometry at 595nm. The values were plotted and compared with the standard graph.

Molecular Identification of Strains by 16s rRNA Sequencing

Among all the isolates that were shown to produce folate, top five producers were identified at molecular level by their 16S rRNA Sequencing. Those isolates were subjected for DNA extraction and purification followed by PCR amplification and sequencing. These 5 isolates were amplified by PCR using universal primers. The primer pairs used for the amplification of 16S rRNA gene was

[F AGA GTT TGA TCM TGG CTC AG -27F],

[R CGG TTA CCT TGT TAC GAC TT -1492R].

The amplicons produced were subjected for agarose gel electrophoresis on 1% agar. 1Kb marker in TAE buffer was used in the gel. After the gel run the amplicons were subjected for washing with sodium acetate and elution with 70% ethanol. Their sequencing was carried out on ABI 3730XL sequencer using Sanger's sequencing method. The assembled DNA sequences obtained were further analyzed using BLAST for identification of the bacteria. The sequence data was also used to construct the phylogenetic tree using Clustal omega, an online bioinformatics tool.

RESULTS AND DISCUSSIONS

Fermented traditional foods are known to be good source of LAB since long. Screening LAB for their beneficial metabolites such as folate is highly desirable as microbial folates are reported to be safe for human health unlike chemically synthesized folic acid.

Bacterial Screening & Identification

In this study fermented food samples were selected as the sources for the isolation of required folate producing bacteria. Based on the variation in the cell morphology, color and culture morphology in the media pure cultures of 50 isolates were prepared on MRS agar plates and each culture was quadrant streaked onto these plates. Among them 42were identified to be Gram positive and catalase negative and thus considered as presumptive Lactic Acid Bacteria. Those 42 cultures were inoculated in FAC medium to confirm their ability to produce folate.

Out of 42 colonies screened for folate production 27 were found to be positive because of turbidity obtained in media.20 positive colonies were randomly selected from these 27 and further analyzed for sugar utilization capacity.

Table 1: Number of Folate Producing Isolates from Fermented Food Samples

Sample	No. of Folate +ve Isolates
Khoa	7
Olives	3
Dosa batter	7
Date syrup	1
Fermented dough	5
Fermented Ragi	4
Total	**27**

Table 2: Carbohydrate Utilization Assessment of Folate Producers

Sample No.	Source	Colony Morphology	Cell Morphology	Glucose	Mannitol	Lactose	Mannose	Maltose	Sucrose	Fructose
1	Khoa	Very small, white, elevated colonies	Bacillus	p	p	p	p	p	n	p
2	Khoa	Very small microscopic colonies, dull cream, smooth	Bacillus	p	p	p	p	p	n	n
3	Khoa	Very small, Round, shining beige colonies	Bacillus	p	n	p	n	n	n	p
4	Khoa	Very small, Round, shining beige colonies	Bacillus	p	n	p	n	n	n	p

Table 2: Contd.,

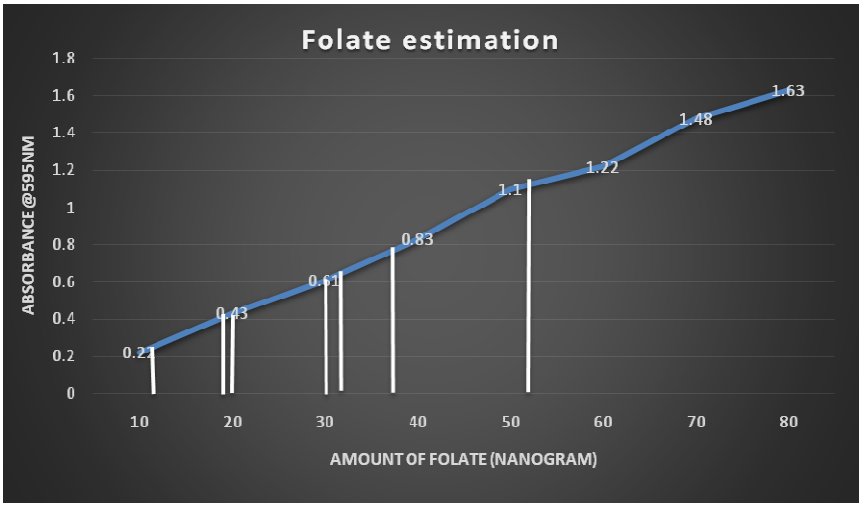
5	Khoa	Grey smooth, convex, shiny, circular colonies	Coccus	n	p	n	p	p	p	p
6	Olives in Brine	Elevated, creamy slimy colonies with smooth ends	Bacillus	p	n	p	p	p	n	n
7	Olives in Brine	Slimy, transparent colonies with smooth elevation	Bacillus	p	p	p	p	p	n	n
8	Dosa batter	Very small microscopic colonies, dull cream, smooth.	Bacillus	p	p	p	p	p	n	n
9	Dosa batter	Slimy cream colonies with smooth ends	Bacillus	p	n	p	n	p	p	p
10	Dosa batter	Slimy cream colonies with smooth ends	Bacillus	p	n	p	n	p	p	p
11	Dosa batter	Grey smooth, convex, shiny and circular colonies.	Coccus	n	p	n	p	p	p	p
12	Dosa batter	Very small microscopic colonies, dull cream, smooth.	Bacillus	p	p	p	p	p	n	n
13	Date	Cream Color elevated colonies with smooth ends. Watery appearance in nature.	Bacillus	n	p	p	p	p	p	p
14	Fermented Sour dough	Cream Color elevated colonies with smooth ends. Watery appearance in nature.	Bacillus	n	p	p	p	p	p	p
15	Fermented Sour dough	Cream color, rough end, serrated colonies bigger colony size	Bacillus	p	n	p	n	p	n	p
16	Fermented Sour dough	Cream Color elevated colonies with smooth ends. Watery appearance in nature.	Bacillus	n	p	p	p	p	p	p
17	Fermented Sour dough	Cream Color elevated colonies with smooth ends. Watery appearance in nature.	Bacillus	n	p	p	p	p	p	p
18	Fermented ragi	white, convex, shiny colony with irregular edges	Coccus	p	p	p	p	n	n	n
19	Fermented ragi	Grey smooth, convex, shiny and circular colonies.	Bacillus	n	p	n	p	p	p	p
20	Fermented ragi	Very small, Round, shining beige colonies	Bacillus	p	n	p	n	n	n	p

Quantitative Estimation of Folate

To quantify folate from isolated LAB, microbiological assay was carried out. They were found to produce folate in varying quantities. Folate is produced by different strains, as reported previously (Kariluoto et al., 2006; Lin and Young, 2000)Folate production by Streptococcus thermophilus (Rao et al., 1984) and L. bulgaricus (Lin and Young, 2000) have

also been reported.

As per Figure 1 and Table 3 the folate production from the studied cultures was in the range of **20-55 µg/L.** The strains (later identified as *Lactococcus lactis*) obtained in this study were found to produce 55µg/L of folate which is quite promising in the food industry.



Note: *The extrapolation of repeat values of OD are not shown in the graph*

Figure 1: Estimation of Folate Produced by Various Cultures in the Study

Table 3: Concentration of Folate Calculated as per Standard Graph

S. No.	Mean OD	Quantity of Folate as per Graph(ng)	Concentration of Folate(ng/ml)
1	0.20	10	100
2	0.40	20	200
3	0.28	14	140
4	0.42	21	210
5	1.10	55	550
6	0.26	13	130
7	0.92	46	460
8	0.60	30	300
9	0.70	35	350
10	0.70	35	350
11	1.10	55	550
12	0.64	32	320
13	0.22	11	110
14	0.26	13	130
15	0.74	37	370
16	0.40	20	200
17	0.40	20	200
18	0.84	42	420
19	1.04	52	520
20	0.50	25	250

Molecular Identification of Bacteria by 16S rRNA Sequencing

The top five folate producers were subjected for DNA Extraction and amplification and sequencing of their 16S rRNA gene. Ribosomal RNA genes have generally been accepted as potential targets for identification and phylogenetic

analysis of bacteria.

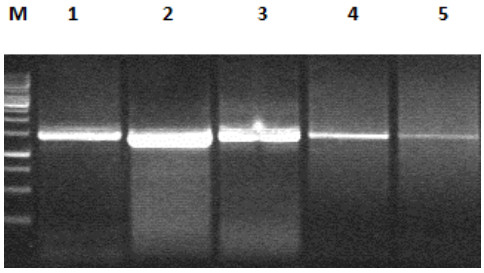
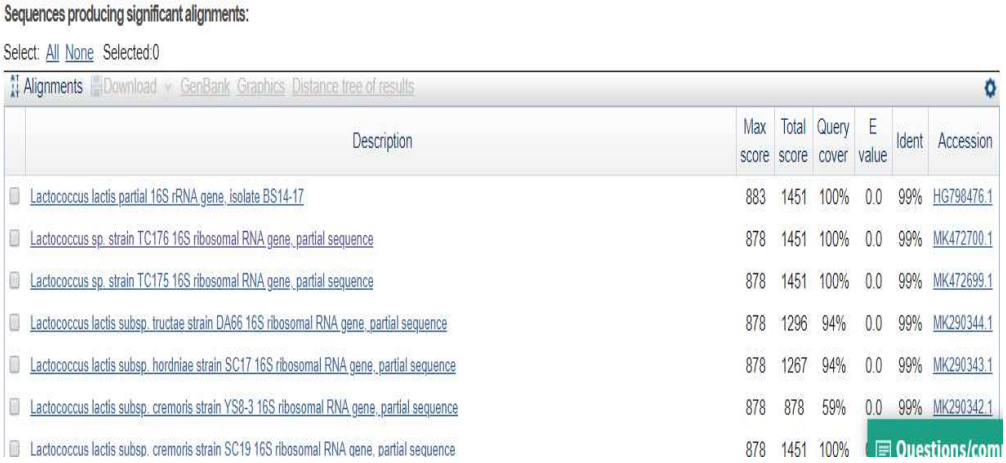


Figure 2: Gel Picture of the 16S rRNA Amplicons

M- 1kb ladder

1-516S rRNA amplicons of the top 5 folate producing isolates

The sequences obtained were searched for similarity in the database by BLAST and further phylogenetic tree of close sequences were constructed. Each of the 5 selected bacteria was compared with its sequence homologues. The results of BLAST and phylogenetic tree identified them as*Lactococcus lactis strain BS14-17, Bifidobacterium bifidum strain NCTC13001*, *Lactococcus lactis subsp. cremorisstrain* YS8-4, *Bifidobacterium lemurum* and*Lactococcus lactis strain Cp1*.



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Lactococcus lactis partial 16S rRNA gene, isolate BS14-17	883	1451	100%	0.0	99%	HG798476.1
Lactococcus sp. strain TC176 16S ribosomal RNA gene, partial sequence	878	1451	100%	0.0	99%	MK472700.1
Lactococcus sp. strain TC175 16S ribosomal RNA gene, partial sequence	878	1451	100%	0.0	99%	MK472699.1
Lactococcus lactis subsp. tructae strain DA66 16S ribosomal RNA gene, partial sequence	878	1296	94%	0.0	99%	MK290344.1
Lactococcus lactis subsp. hordniae strain SC17 16S ribosomal RNA gene, partial sequence	878	1267	94%	0.0	99%	MK290343.1
Lactococcus lactis subsp. cremoris strain YS8-3 16S ribosomal RNA gene, partial sequence	878	878	59%	0.0	99%	MK290342.1
Lactococcus lactis subsp. cremoris strain SC19 16S ribosomal RNA gene, partial sequence	878	1451	100%			

Figure 3: The Blast Output and Phylogenetic Tree of 16S rRNA Sequence of Lactococcus Lactisst BS14-17

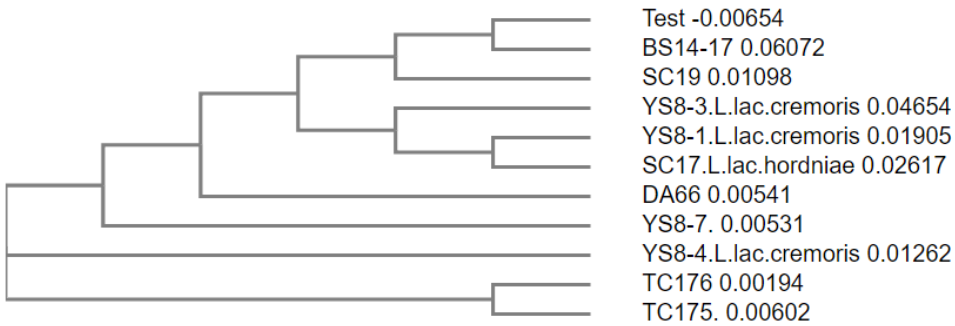


Figure 4: The Blast Output and Phylogenetic Tree of 16S rRNA Sequence of Bifido Bacterium Bifidum Strain NCTC13001

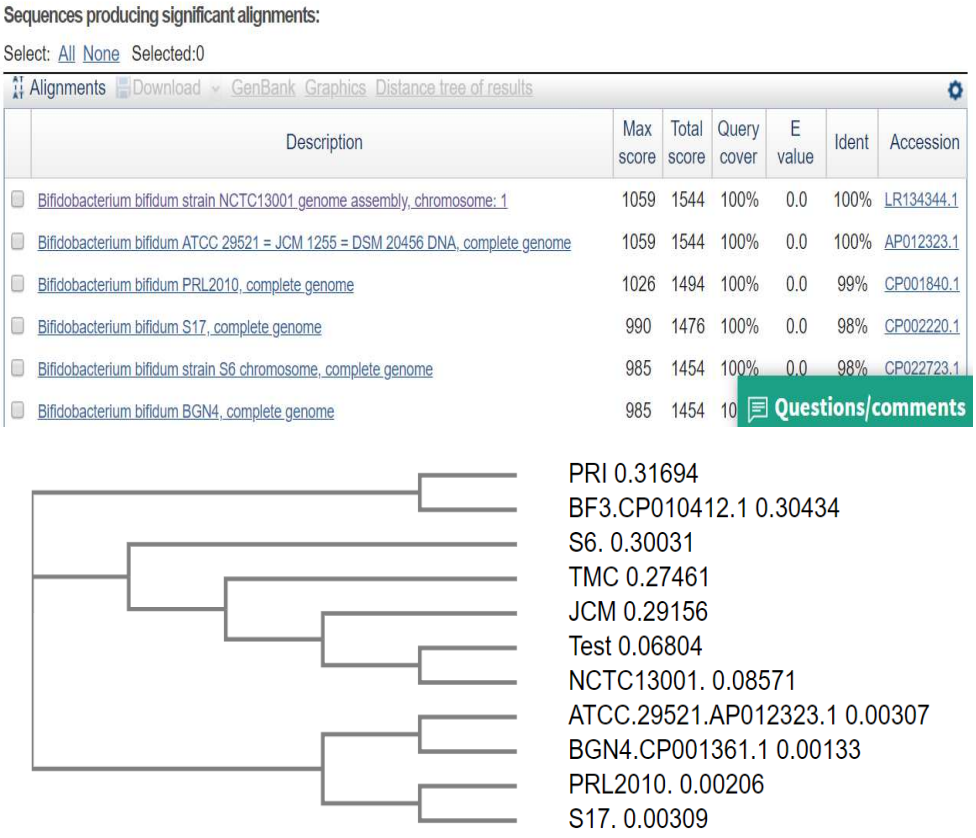


Figure 5: The Blast Output and Phylogenetic Tree of 16S rRNA Sequence of *Lactococcus Lactis Subsp. Cremoris* Strain YS8-4

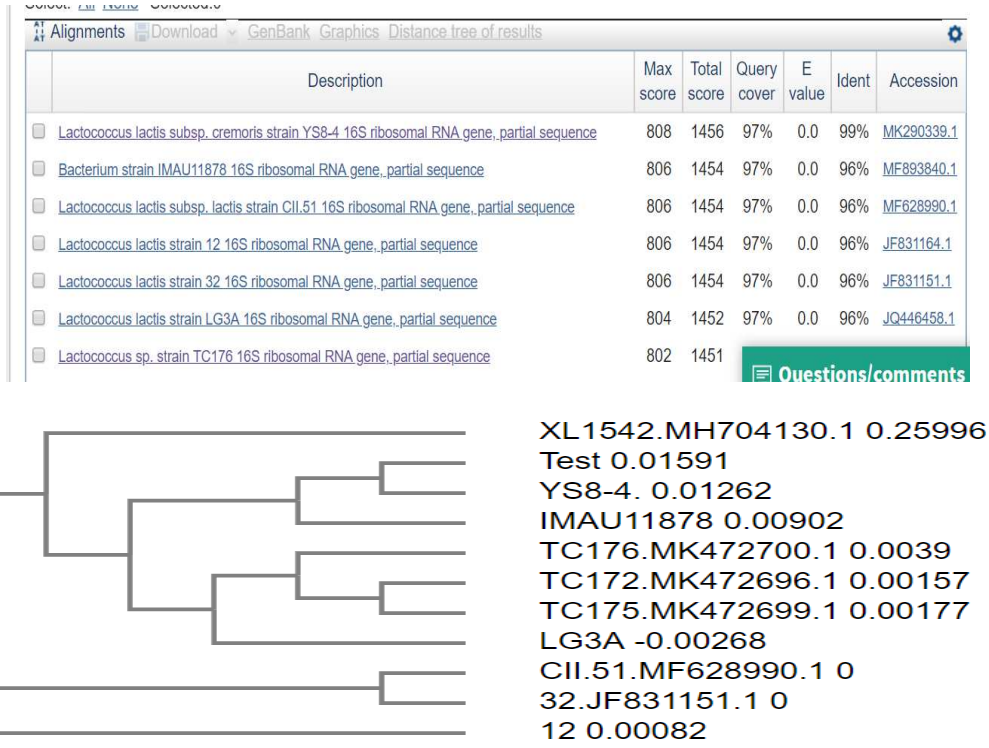


Figure 6: The Blast Output and Phylogenetic Tree of 16S rRNA Sequence of Bifidobacterium Lemurum

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bifidobacterium lemurum 16S ribosomal RNA, partial sequence	1548	1548	100%	0.0	99%	NR_135862.1
Bifidobacterium eulemuris strain LMM_E3 16S ribosomal RNA, partial sequence	1543	1543	100%	0.0	99%	NR_148784.1
Bifidobacterium eulemuris strain LMM_E3 16S ribosomal RNA gene, partial sequence	1543	1543	100%	0.0	99%	KP979748.1
Bifidobacterium sp. LMM_I9 16S ribosomal RNA gene, partial sequence	1533	1533	100%	0.0	99%	KU171117.1
Bifidobacterium breve strain lw01 chromosome, complete genome	1432	2864	100%	0.0	97%	CP034192.1
Bifidobacterium breve strain NRBB56 chromosome, complete genome	1432	2864	100%	0.0	97%	CP021394.1
Bifidobacterium pullorum gene for 16S ribosomal RNA, partial sequence, strain: JCM 1214	1432	1432	100%	0.0	97%	LC071802.1

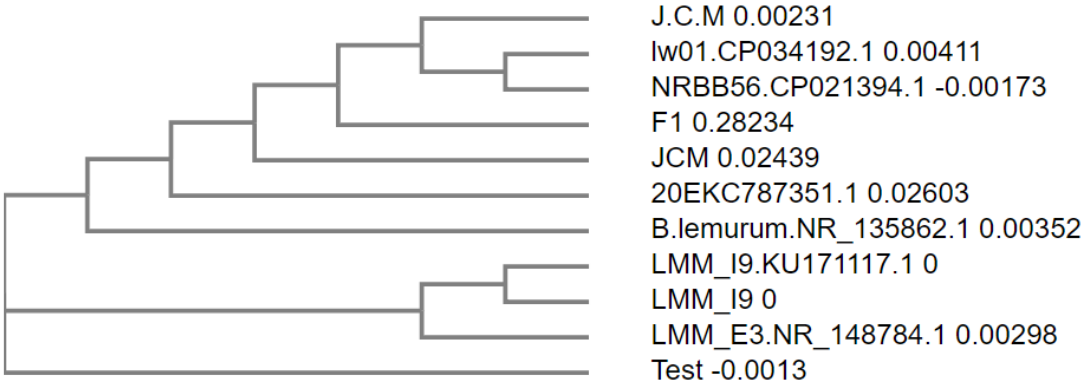


Figure 7: The Blast Output and Phylogenetic Tree of 16S rRNA Sequence of *Lactococcus Lactis Strain Cp1*

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Lactococcus lactis strain Cp1 16S ribosomal RNA gene, partial sequence	1474	1474	100%	0.0	98%	MH295792.1
Lactococcus lactis partial 16S rRNA gene, isolate isolate 1	1474	1474	100%	0.0	98%	HF677501.1
Bacterium strain IMAU11950 16S ribosomal RNA gene, partial sequence	1461	1461	100%	0.0	98%	MF893873.1
Bacterium strain IMAU11843 16S ribosomal RNA gene, partial sequence	1461	1461	100%	0.0	98%	MF893824.1
Bacterium strain IMAU11816 16S ribosomal RNA gene, partial sequence	1461	1461	100%	0.0	98%	MF893801.1
Lactococcus sp. BYMS21 16S ribosomal RNA gene, partial sequence	1461	1461	100%	0.0	98%	KU891844.1
Uncultured Lactococcus sp. gene for 16S ribosomal RNA, partial sequence, clone: Co1-26	1461	1461	100%	0.0	98%	AB749376.1
Uncultured Lactococcus sp. gene for 16S ribosomal RNA, partial sequence, clone: A2-12	1461	1461	100%	0.0	98%	AB750693.1
Lactococcus lactis subsp. lactis partial 16S rRNA gene, strain ZG6-51	1461	1461	100%	0.0	98%	HE646424.1

Figure 8: Phylogenetic Tree Constructed among Obtained Strains and Their Closet Homologues

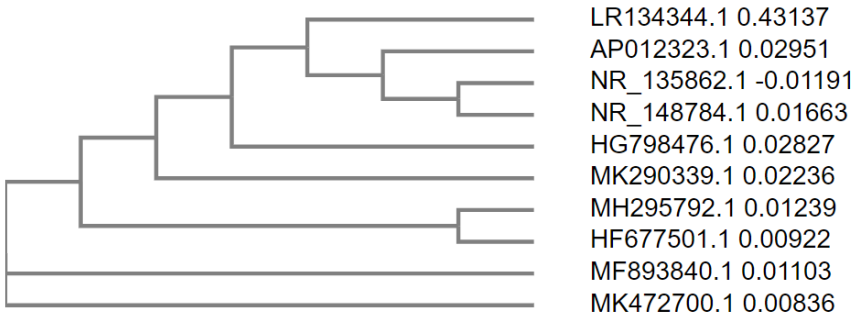


Figure 9

DISCUSSIONS

As folate is an essential vitamin which needs to be supplied by external food source and cannot be synthesized in the body, This can prevent the nutrition deficiency disorders. The current work includes the isolation and identification of folate producing bacteria from fermented foods for the use of industrial scale food fortification. Use of microbial folate is both economic and healthy. From the bacteria isolated and studied, 5 best producers have been identified to be of Lactococcus sp and Bifidobacterium species. This research also includes the phylogenetic study of these 5 selected bacteria which can be an insight to the other bacteria which could be used as folate producers in the bio fortification of food products. The study concludes that bifidobacterium and Lactococcussp are the best producers of folate. It is expected that by using the five isolated strains of this study in combination with specific growth conditions, the current intake of folate from selected food products can be enhanced. Incorporation of such potential bacteria in commercial functional foods confer health benefits to the consumers. As such they can be further studied at large scale as an alternative to synthetic folates. They could be further exploited for biofortification studies in different food matrices either as individual or mixed cultures.

CONCLUSIONS

The study concludes that Bifidobacterium and Lactococcus sp. are the best producers of folate. It is expected that by using the five isolated strains of this study in combination with specific growth conditions, the current intake of folate from selected food products can be enhanced. Incorporation of such potential bacteria in commercial functional foods confer health benefits to the consumers. As such they can be further studied at large scale as an alternative to synthetic folates. They could be further exploited for biofortification studies in different food matrices either as individual or mixed cultures. Their invitro studies can also be carried out to evaluate their probiotic properties such as growth in bile, gastric juice, their antibacterial character towards human pathogens so as to utilize them in advanced research studies.

REFERENCES

1. *Alm, L. 1980. Effect of fermentation on B-vitamin content of milk in Sweden. J. Dairy Sci. 65:353-359.*
2. *Arth A, Kancherla V, Pachn H, Zimmerman S, Johnson Q, Oakley GP Jr. (2016) A 2015 global update on folic acid-preventable spina bifida and anencephaly. Birth Defects Res A ClinMolTeratol. 106(7) 520-9. doi: 10.1002/bdra.23529.*
3. *Baggott, J. E., Oster, R. A., & Tamura, T. (2012). Meta-analysis of cancer risk in folicacid supplementation trials. Cancer Epidemiology, 36(1), 78e81. doi:S1877-7821(11)00084-1 [pii] 10.1016/j.canep.2011.05.003.*
4. *Becker W, Lyhne N, Pederson A N, Aro A, et al.(2004) Nordic nutrition recommendations 2004- Integrating nutrition and physical activity. Scandinavian journal of food and nutrition 48 178-187.*
5. *Bernardeau M., Guguen M., Vernoux J. P. (2006). Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. FEMS Microbiol. Rev. 30.*
6. *Camilo, E., J. Zimmerman, J. B. Mason, B. Golner, R. Russell, J. Selhub, and I. H. Rosenberg. 1996. Folate synthesized by bacteria in the human upper small intestine is assimilated by the host. Gastroenterology 110:991-998.*
7. *Crittenden, R. G., Martinez, N. R. and Playne, M. J. 2003. Synthesis and utilization of folate by yoghurt starter cultures and probiotic bacteria. International Journal of Food Microbiology 80: 217– 222.*
8. *Coppen A, Bailey J. Enhancement of the antidepressant action of fluoxetine by folic acid: a randomised, placebo controlled trial. J Affect Disord. 2000;60:121–30.*
9. *Dudeja, P. K., S. A. Torania, and H. M. Said. 1997. Evidence for the existence of a carrier-mediated folate uptake mechanism in human colonic luminal membranes. Am. J. Physiol. 272:G1408-G1415.*
10. *J. Durga, M. P. van Boxtel, E. G. Schouten, F. J. Kok, J. Jolles, M. B. Katan and P. Verhoef, Lancet. 369. 9557.*
11. *FAO/WHO, in: Human vitamin and mineral requirements, Bangkok, Thailand, (2002).*
12. *Guarner, F, Scaafsma G. J. Probiotics. Int. J. Food Microbiol, 1998; 39:2.*
13. *Hanson AD, Gregory JF. 2011. Folate biosynthesis, turnover, and transport in plants. Annu Rev Plant Biol. 62(1):105-125.*
14. *Horne, D. W., & Patterson, D. (1988). Lactobacillus casei microbiological assay of folic acid erivatives in 96-well microtiter plates. Clinical Chemistry, 34(11), 2357-2359.*
15. *Hugenholtz, J., Hunik, J., Santos, H., &Smid, E. (2002). Nutraceutical production by propionibacteria. Le Lait, 82, 103–112.*
16. *Iyer R, Tomar SK. Folate: a functional food constituent. J Food Sci 2009;74:114–22.*
17. *Kariluoto, S., Aittamaa, M., Korhola, M., Salovaara, H., Vahteristo, L., &Piironen, V. (2006). Effects of yeasts and bacteria on the levels of folates in rye sourdoughs. International Journal of Food Microbiology, 106(2), 137e143. doi:S0168-1605(05) 00422-8 [pii] 10.1016/j.ijfoodmicro.2005.06.013.*
18. *Katan MB, Boekschoten MV, Connor WE, Mensink RP, Seidell J, Vessby B et al. 2009. Which are the greatest recent discoveries and the greatest future challenges in nutrition? Eur J Clin Nutr 63:2-10.*
19. *Sreejith, K., & Sebastian, C. D. (2014). Molecular evolutionary analysis of paddy pest, Cofana spectra (Distant)(Hemiptera: Cicadellidae) using partial DNA sequence of cytochrome oxidase subunit I (COI) gene. IJANS, 3(2), 135-40.*
20. *Kumar, C. K., M. P. Moyer, P. K. Dudeja, and H. M. Said.1997. A protein-tyrosine kinase-regulated, pH-dependent, carrier-mediated uptake system for folate in human normal colonic epithelial cell line NCM460. J Biol Chem. Mar 7;272(10):6226-31.*

21. *Konings, E. J., Roomans, H. H., Dorant, E., Goldbohm, R. A., Saris, W. H., & van den Brandt, P. A. (2001). Folate intake of the Dutch population according to newly established liquid chromatography data for foods. The American Journal of Clinical Nutrition, 73(4), 765e776.*
22. *Krause, L. J., C. W. Forsberg, and D. L. O'Connor. 1996. Feeding human milk to rats increases Bifidobacterium in the cecum and colon which correlates with enhanced folate status. J. Nutr. 126:1505-1511.*
23. *Laiño, J. E., Juarez del Valle, M., Savoy de Giori, G., & LeBlanc, J. G. J. (2013). Development of a high folate concentration yogurt naturally bio-enriched using selected lactic acid bacteria. LWT– Food Science and Technology, 54, 1–5*
24. *LeBlanc JG, Giori GSD, Smid EJ, Hugenholtz J and Sesma F. Folate production by lactic acid bacteria and other food-grade microorganisms. Communicating Current Research and Educational Topics and Trend in Applied Microbiology 2007; 1, 329–39.*
25. *Lin, M. Y., and C. M. Young. 2000. Folate levels in cultures of lactic acid bacteria. Int. Dairy J. 10:409-413.*
26. *Luchsinger, J. A. M. X. Tang, J. Miller, R. Green and R. Mayeux, Arch 64. 1. 86 (2007).*
27. *Melnyk, S., Pogribna, M., Miller, B. J., Basnakian, A. G., Pogribny, I. P., & James, S. J.(1999). Uracil misincorporation, DNA strand breaks, and gene amplification are associated with tumorigenic cell transformation in folate deficient/repleted Chinese hamster ovary cells. Cancer Letters, 146(1), 35e44. doi: S030438359900213X [pii]. Murdock, F. A., & Fields, M. L.*
28. *Moreno, F., Sarantinopoulos, M. R., Tsakalidou, P. E., De Vuyst, L. (2006). The role of application of Enterococci in food and health. International Journal of Food Microbiology. 106: 1-24.*
29. *Patel A., Cecilia L., Arti P., Prajapati J. B., Olle H. 2012. Probiotic properties of exopolysaccharide producing lactic acid bacteria isolated from vegetables and traditional Indian fermented foods. International Journal of Fermented Foods 1:87-101.*
30. *Das, M. K. (2014). Starter culture development of sindol-a fermented fish product of Assam. International Journal of Agricultural Science and Research, 4(2), 107.*
31. *Rao, D. R; Reddy, A. V; Pulusani, S. R; & Cornvel, P. E;(1984) Biosynthesis & utilization of folic acid and vitamin B12 by lactic cultures in skim milk. Journal of dairy science,67,1169-1174.*
32. *Refsum H, Smith AD. Are we ready for mandatory fortification with vitamin B-12 American Journal of Clinical Nutrition 2008;88:253–4.*
33. *Rossi, M., Amaretti, A. and Raimondi, S. 2011. Folate production by probiotic bacteria. Nutrients 3: 118- 134.*
34. *Said, H. M., and Z. M. Mohammed. 2006. Intestinal absorption of water-soluble vitamins: an update. Curr. Opin. Gastroenterol. 22:140-146.[*
35. *Saini RK, Nile SH, Keum YS. Folates: chemistry, analysis, occurrence, biofortification and bioavailability. Food Res Int. 2016;89(Pt 1):1–13. doi: 10.1016/j.foodres.2016.07.013.*
36. *Shane B. Folate fortification: enough already? American Journal of Clinical Nutrition 2003;77:8–9.*
37. *Singh et. al. Folate, vitamin B12, ferritin and hemoglobin levels among women of childbearing age from a rural district in South India. BMC Nutrition 2017, 3(50). https://doi.org/10.1186/s40795-017- 0173-z*
38. *Smith AD. 2008. The worldwide challenge of the dementias: A role for B vitamins and homocysteine? Food Nutr Bull Suppl 29:143-172.*
39. *Stamer, J. R. 1979. The lactic acid bacteria : Microbes of diversity. Journal Food Technology : 60-65.*

40. *Sharma, Nivedita., Handa, Shweta., & Gupta, Anupama. (2013). Comprehensive study of different traditional fermented foods/beverages of himachal pradesh to evaluate their nutrition impact on health and rich biodiversity of fermenting microorganisms. International Journal of Research in Applied Natural and Social Sciences, 1, 19-28.*

41. *Ulrich, C. M., & Potter, J. D. (2006). Folate supplementation: too much of a goodthing? Cancer Epidemiology, Biomarkers & Prevention, 15(2), 189e193. doi:15/2/189 [pii]10.1158/1055-9965.EPI-152CO.*

42. *Vijai P., Marilingappa J. &Kadirvelu J. (2005). Isolation and characterization of bacteriocins producing lactic acid bacteria from a South Indian special Dosa (Appam) batter. Journal of Culture Collection. 4, 53-60.*